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Research Article

ANALYSIS AND IDENTIFICATION OF MICROBIAL SPECIES ASSOCIATED WITH THE EGYPTIAN DATE FRUITS DURING POSTHARVEST STORAGE

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ABSTRACT. Date fruits are consumed in many countries globally and are mostly loaded with a mixture of microbes. As a result, various bacterial and fungal species attack them, thus causing their spoilage at the ripening, storage, and processing stages. The present study investigates the current spoilage bacteria and fungi of twelve postharvest date fruits collected from different Egyptian geographical regions (Aswan, Siwa, and El-Wady El-Gadeed). Isolation and identification of date-borne microbes based on morphological and molecular identification were studied. The microscopic examination of infected date samples showed various fungal structures (e.g., hyphae, spores, conidial head, etc.). In addition, all fungal structures observed in the infected specimens belong to the fungus *Aspergillus niger.* After six days of incubation on a PDA medium, the total viable counts of fungi in twelve date varieties samples ranged from 1500 to 80000 (CFU/g). Five fungal isolates were isolated (*Aspergillus niger* F4, *A. terreus* F1, *Penicillium* sp. F3, *Rhizopus* sp. F2, and *Circinella* sp. F5) from the different samples. *A. niger* F4 (GenBank accession number MW811390) was the most abundant in all samples. Similarly, the total viable bacteria counts in twelve date varieties samples ranged from 4500 to 75000 (CFU/g) after 48 h incubation on nutrient agar (NA) medium. Four morphologically different bacterial phenotypes (B1, B2, B3, and B4) were isolated and purified on the NA plates. *Pseudomonas aeruginosa* B1 (GenBank accession number MW811391) was the most abundant bacterial isolate in all samples. Aflatoxins were also analyzed using HPLC/MS/MS in all date varieties. The results showed that aflatoxins G2, G1, and B2 were not detected in all date varieties. However, aflatoxin B1 was detected in two varieties, Partamoda and Frehi.

Keywords: *Date fruits, microbial analysis, total viable counts, aflatoxins analysis.*

INTRODUCTION

Date fruits of the date palm (*Phoenix dactylifera* L.) are the main staple food source in arid and semi-arid regions of North Africa, the Middle East and South-Asian countries [1]. Consequently, dates have always played an essential role in the economic and social lives of people of this area. Date fruit is a highly nutritious food product, rich in calories and many vitamins and minerals [2, 3]. Some date varieties ripen early in the season. However, other types do not mature until the end of the season. Due to variable growing

conditions and genetic differences, dates vary significantly in their final appearance and sensory, physical, and chemical properties [4]. Many products, quality control systems, process equipment designs, shelf life prediction models, packaging and storage could be developed using these parameters.

Like most agricultural products, dates are prone to preharvest and postharvest mold infestation by specific pathogens [5, 6]. Contamination by microbes, especially fungi, is a big problem for international marketing [7, 8]. Date fruits are consumed in many countries globally and are mostly loaded with a mixture of microbes. At ripening and during storage and processing, they are attacked by numerous bacterial and fungal species, which cause the fruit to spoil [9, 10 ,11, 12]. The microorganisms growing on preharvest and postharvest stored products can cause damage resulting in a reduction in the quality and quantity of the fruits. In addition, many bacterial and fungal species are capable of producing mycotoxins.

Mycotoxins and mycotoxigenic fungi probably invaded human food thousands of years ago. As a result, the identities of mycotoxins have only been discovered in recent decades since aflatoxins were discovered [13]. It has been described that mycotoxins can affect human health in a variety of ways, including liver diseases, hemorrhaging, renal dysfunction, hepatic failure, immune suppression, liver and esophageal cancer, reproductive disorders, and even death in case of acute intoxication [14, 15]. There are three principal fungi that produce mycotoxins: *Aspergillus* species (produces aflatoxins), *Penicillium* species (produces ochratoxin), and *Fusarium* species (produces T-2, HT-2, deoxynivalenol, nivalenol, zearalenone, and fumonisins). [6, 16]. Even a single fungus can produce a variety of secondary metabolites with different molecular weights and structures. For instance, *A. niger* species are thought to be able to produce both ochratoxin A (OTA) and fumonisin B2 (FB2) [17]. High concentrations of various sugars, minerals, vitamins, and amino acids, and low pH also enhance the growth of different parasitic and saprophytic fungi and the production of mycotoxins [18]. Therefore, there is a need to identify these microorganisms, especially those pathogenic to humans, to reduce the risk of contamination and infection from handling and consuming fruits [19, 20].

A variety of postharvest date fruits collected from different Egyptian geographical regions (Aswan, Siwa, and El-Wady El-Gadeed) were examined in this study to assess variation in composition and microbiological quality, as well as to establish a bacterial and fungal profile for fruits. Isolation and identification of dates-borne microbes based on morphological and molecular identification of the most common microbes were investigated. In addition, a quantitative analysis of the natural occurrence of aflatoxins in dried date fruits was performed.

MATERIALS AND METHODS

Chemicals, reagents, and microbiological media

Microbiological media, including potato dextrose agar (PDA), nutrient agar (NA), nutrient broth (NB), and potato dextrose broth (PDB), were purchased from Oxoid Ltd (Basingstoke, Hampshire, UK). An antibiotic, chloramphenicol (99%), was obtained from Pharco Pharmaceuticals Inc. (Alexandria-Cairo Desert Road, Amriya, Alexandria, Egypt). The high-purity solvents, including acetone, acetonitrile, methanol, chloroform, dimethyl sulphoxide (DMSO), and trifluoroacetic acid, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The standards of aflatoxins (B1, B2, G1,

and G2) were purchased from Sigma Aldrich, Steinheim, Germany. Other solvents and chemicals were purchased from El-Gomhoria for Pharmaceutical and Chemicals Co. (Alexandria, Egypt) and used without further purification.

Sampling of date fruits

Twelve common date fruits (*Phoenix dactylifera* L.) varieties were obtained from three Egyptian geographical regions; Aswan, Siwa and El-Wady. Six dry varieties were obtained from Aswan, including Aynate, Ghazaly, Melkaby, Partamoda, Shamea, and Wardy. Four dry varieties were obtained from Siwa, including Azzawi, Frehi, Kaeiby, and Shamea. In addition, two semi-dry varieties, El-Wady I and El-Wady II, were obtained from of El-Wady El-Gadeed region. Dry and semi-dry date fruit samples (10 kg) were chosen randomly, taken in sterile polyethylene bags, and transported to the laboratory for microbiology analysis [10].

Fruit quality analysis

Following distilled water washing of dates, the biochemical composition was determined. The moisture content (humidity), protein, fat, fiber, ash content, and soluble carbohydrates were determined, and the results are expressed as a percent of wet basis. The moisture content was determined using 5 g of pulp spread out in a tared stainless capsule and then dried in an oven at 105 °C for 24 h until a constant weight was reached [21]. The micro Kjeldahl's method determined the total protein content on a dry weight basis of the date samples [22]. The percentage of crude protein was calculated as % N×4.38 [23]. Fat content was determined by AOAC [22] method. The extraction was done by a mixture of chloroform and methanol (2:1, respectively). Fat content was expressed as a percentage. Total dietary fiber content was determined according to the AOAC method [24]. Date flesh was treated in hot water at 70 °C (10g/60 mL) for 15 minutes before extracting fibres. The insoluble residue was then separated from the mixture by filtering. Fiber extracts were dried at 100°C in the oven, and the product was weighted. Ash content was determined after heating macerated samples of date in a muffle oven for 8 h at 600 °C. Carbohydrate (sugars) content was extracted from macerated dates by water at 85 °C and was quantified using the 3,5-dinitrosallicylic acid method [25].

Microbial analysis
Sample preparation

Firstly, date fruit samples were examined with the naked eye. Observably infected samples were analyzed directly by a light microscope. A 10 g sample of each date was aseptically weighed into sterile stomacher bags and then mixed with 200 mL sterile distilled water. After homogenizing the samples, an aliquot (0.1 mL) was used to perform the microbiological analysis [10].

Isolation and purification of dates-borne microbes

Fungi were isolated and enumerated by pour plate technique from dates using PDA plates (three replicates) supplemented with 100 µg/mL of chloramphenicol to inhibit bacterial growth. Plates were inoculated by 0.1 mL aliquots of homogenate dilution and incubated at 28 °C for 6 days. Similarly, bacteria were isolated and enumerated by the

NA plates technique [12]. The plates were inoculated with 0.1 mL aliquots of homogenate dilution and incubated at 28 °C for 48 h. The morphologically different colonies were selected for purification. Micro colonies were purified by repeated streaking on PDA and NA plates and incubating at 28 °C for 2-6 days [10]. In addition, the infected samples of dates were into 3 mm pieces with a sterile razor blade and surface-sterilized in 1% hypochlorite for 2 minutes. The samples were placed on the PDA or NA and incubated at 28 °C for 6 days for fungi and 28 °C for 1-2 days for bacteria. After incubation, colonies of different shapes and colours were observed on the plates. The pure colonies obtained were transferred to fresh slants, subcultured and stored at 4°C. Bacterial and fungal colonies were counted and expressed as colony-forming units per gram of the date fruits (CFU/g).

Identification of dates-borne microbes
Morphological identification

The fungi were identified based on the morphological characteristics after culturing on the PDA media at 28 °C for 7 days. The cultures were identified based on macroscopic and microscopic features. The fungal isolates were identified as described by (Moubasher, 1993) based on morphological characteristics. In addition, the bacteria were identified based on the colony morphology characteristics such as size, appearance, motility, configuration, and elevation on the NA plates after 24 h at 28 °C [26]. Cell morphology was also microscopically examined after Gram staining of 24 h old cultures grown on NA slants. Spore formation was examined microscopically using endospore staining of cells from 48 h hold colonies.

Molecular identification of the most common microbes

Molecular characterizations were carried out at the Sigma Co. lab in Cairo, Egypt. For molecular identification of the dominant fungal isolate (designated as F4), fungal genomic DNA extraction was made according to the procedure of Quick-DNA™ Fungal Microprep Kit's procedure. The internal transcribed spacer (ITS) region of rDNA was PCR amplified using the primers set of ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') [27] according to the procedure of Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Fisher Scientific Inc.). The size and purity of the amplified PCR products were checked by gel electrophoresis. The amplified PCR products were sequenced on both strands using ITS1 and ITS4 primers using an ABI 3730xl DNA sequencer. Similarly, for molecular identification of the most common bacterium (designated as B1), genomic DNA extraction was carried out with Gene Jet genomic DNA preparation kit (Omega Bio-Tek, USA). The 16S rDNA was incompletely amplified with the primers pairs of (16S forward primer; 8F) 5′-AGAGTTTGATCCTGGCTCAGG-3′ and (16S reverse primer; 1492R) 5′-ACGGCTACCTTGTTACGACTT-3′. The PCR program was as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 15 min. The PCR reaction components were 20 μM of primer, 2.5 mM dNTPs, 50 mM MgCl2, 10×Taq buffer, 100 ng sample DNA, 2.5 U Taq DNA polymerase enzyme and distilled water mixed in a final volume of 100 μL. Gel electrophoresis was carried out to confirm the size and purity of the amplified PCR products. The PCR amplification products were sequenced, on both strands, using Perkin Elmer ABI 377 sequencer and a Taq FS Dye Terminator Sequencing Kit (ABI, USA) [28].

The nucleotide sequences obtained were compared with those sequences already deposited in the data bank of the National Centre for Biotechnology and Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the nucleotide basic local alignment search tool (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the most closely related sequences. The identification of the species was determined based on the best sequence alignment score. The nucleotide sequences determined were deposited in GenBank.

Quantitative analysis of aflatoxins in date samples by HPLC/MS/MS

The date samples were extracted using a solvent mixture of acetonitrile/water/acetic acid (79:20:1, v/v/v) and shaken using a rotary shaker for 90 min in a horizontal position. The supernatant (300 µL) was transferred into HPLC vials and diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v). A quantitative analytical method of the aflatoxins was performed using HPLC equipped with a mass spectrometer (LC/MS/MS, QCAP, Central Laboratory for analysis of pesticide residues and heavy metals in food, Egyptian Ministry of Agriculture, 7 Nadi El-said street, Dokki, Giza, Egypt) [29]. Briefly, a QTrap 5500 MS/MS system (Sciex, Foster City, CA, USA) equipped with a TurboV electrospray ionization (ESI) source was coupled to a 1290 series HPLC system (Agilent Technologies, Waldbronn, Germany). Five µL of the diluted extract was injected into the LC-MS/MS system without further pre-treatment. Chromatographic separation was performed at 25 °C on a C18-column, 150×4.6 mm i.d., 5 µm particle size, equipped with a C18 security guard cartridge, 4×3 mm i.d. (Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode with 1 mL/min flow rate. Methanol/water/acetic acid mixtures 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B) contained 5 mM ammonium acetate in both of the mobile phases. A linear increase in the proportion of B to 50% was achieved within three minutes after an initial 2 min period at 100% A. In the next 9 min, B was linearly increased to 100% and was held for 4 min at this level, followed by 2.5 min of column re-equilibration at 100% A. A volume of 5 µL was injected. ESI-MS/MS was performed in both the positive and negative polarities during two chromatographic runs. The identification is confirmed by checking whether the ion ratio matches the standard values within 30% [30]. In contrast, a stricter in-house criterion of ± 0.03 min is applied for the retention time. The LOQ was also determined.

Statistical analysis

Statistical analysis was performed using the SPSS 25.0 software (SPSS, Chicago, IL, USA) [31]. First, the means and standard errors were calculated and reported. Then, the analysis of variance (ANOVA) of the data was conducted and means property values were separated with Student-Newman-Keuls (SNK) test for the property values. The differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Chemical composition of date flesh

The date flesh for all varieties was characterized by the predominance of moisture content (humidity), protein, fat, fiber, ash content, and soluble carbohydrates (Table 1). The moisture content or humidity ranged from 14.05% for Aynate dates from Aswan to 19.85% for Kaeiby dates flesh from Siwa. No significant differences in crude protein

contents were observed between the twelve varieties. The highest percentage was 3.28% in Wardy date flesh from Aswan, whilst the lowest was found in El-Wady I date flesh (2.37%) obtained from El-Wady El-Gadeed. The crude fat content ranged from 0.17 to 2.24%, which is high to those reported by Sawaya, Khalil [32] for dates grown in Saudi Arabia, Ahmed, Ahmed [33] for dates grown in the United Arabe Emirate; Hasnaoui, ElHoumaizi [34] for different date verities from Morocco, and those of some Iranian varieties (0.10 to 0.9% of fat) [35]. Fat is mainly concentrated in the skin (2.5 to 7.5%) and has more physiological importance in protecting the fruit than contributing to the nutritional value of the date flesh [36]. The fiber content ranged from 1.41% for Frehi dates from Siwa to 3.35% for Partamoda dates flesh from Aswan. Ash content ranged from 2.16% for Wardy dates flesh from Aswan to 3.07% for Azzawi dates flesh from Siwa. No significant differences in soluble carbohydrates were observed between the twelve varieties, except the Aynate dates from Aswan, which indicated the highest percentage of 87.69%. The other eleven verities presented the range of 70.87-76.16% of soluble carbohydrates. This range is approximately similar to those reported by Hasnaoui, ElHoumaizi [34] (61.64 to 83.32%). The differences in the chemical composition of date flesh may have several causes; firstly, due to varieties. The local and climatic conditions at the time of harvesting will likely affect the final composition of dates flesh.

Table 1. *Chemical composition of different varieties of dates*

Geographical region	Variety of dates	Humidity (%) ± SE	Protein (%) ± SE	Fat (%) ± SE	Fiber (%) ± SE	Ash (%) ± SE	Soluble carbohydrates (%) ± SE
Aswan	Aynate	14.05±0.94	2.87±0.03	0.17±0.03	1.97±0.03	2.25±0.9	87.69±0.47
	Ghazaly	15.43±0.47	2.86±0.38	1.51±0.24	1.76±0.03	2.66±0.3	75.78±0.94
	Melkaby	15.57±1.41	3.02±0.01	1.81±0.01	2.59±0.24	2.17±0.05	74.84±0.94
	Partamoda	15.37±0.14	2.85±0.02	1.91±0.01	3.35±0.14	2.32±0.14	74.20±1.41
	Shamiya	14.11±0.05	2.65±0.28	2.24±0.05	2.41±0.19	2.43±0.0	76.16±0.47
	Wardy	15.29±0.47	3.28±0.09	0.37±0.05	2.88±0.04	2.16±0.05	76.02±0.46
Siwa	Azzawi	18.15±0.05	2.92±0.01	2.17±0.05	1.97±0.03	3.07±0.03	71.72±0.47
	Frehi	17.83±0.94	2.59±0.24	0.22±0.01	1.41±0.19	2.84±0.02	75.56±0.24
	Kaeiby	19.85±0.02	2.42±0.19	1.58±0.24	2.46±0.19	2.82±0.01	70.87±0.47
	Shamiya	18.95±0.47	3.02±0.47	2.09±0.04	1.79±0.33	2.96±0.03	71.17±0.42
El-Wady El-Gadeed	El-Wady I	17.69±0.47	2.37±0.03	2.11±0.03	1.99±0.02	2.19±0.05	73.65±0.47
	El-Wady II	16.91±0.94	2.66±0.03	1.76±0.03	2.08±0.04	2.25±0.05	74.34±0.49

Values are mean of three determinations and given as mean ± standard error (SE).

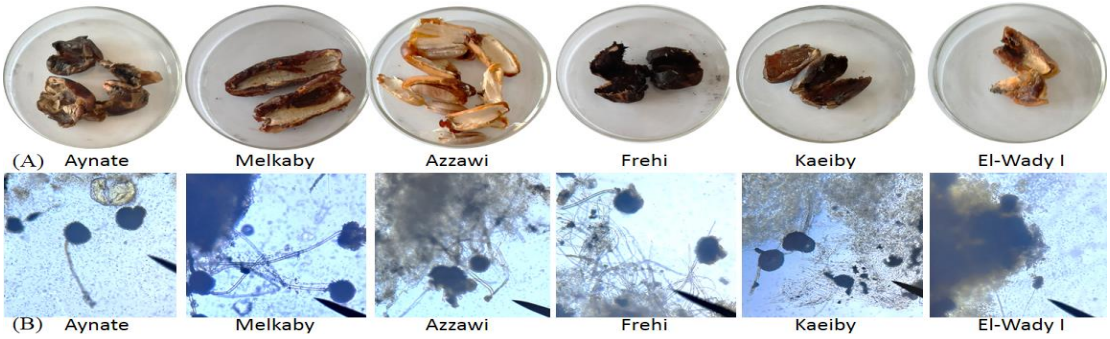

Fig. 1. *The visual appearance of the infected date samples (**A**) and their microscopic slides (**B**) with fungal structure.*

Visual and microscopic examination of the infected date samples

Figure 1A shows the visual observation of the infected date varieties. The infected date samples showed various fungal structures (e.g., hyphae, spores, and conidial head) by applying the microscopic examination. All fungal structures observed in the infected specimens belong to the fungi *Aspergillus* sp. (Figure 1B).

Microbiological analysis

The total viable count of fungi and their distribution among date samples are shown in Table 2. This parameter is an index of the general microbiological quality. The fungal count ranged from high such as El-Wady II (80000 CFU/g) and El-Wady I (64000 CFU/g), to low, such as Ghazaly (1500 CFU/g) and Shamiya from Aswan (2500 CFU/g) after 6 days of incubation on the PDA medium. These results indicate a high level of contamination in some date verities, especially from El-Wady El-Gadeed. The total viable count (CFU/g) of bacteria and their distribution among date samples are also shown in Table 2. The count was significantly high in Aynate date fruits (75000) and El-Wady I (60000). However, the date fruits of Azzawi from Siwa presented the lowest count of bacteria (4500 CFU/g) after 48 h incubation on an NA medium.

Table 2. *Total count and distribution of fungal and bacterial isolates among date samples*

Geographical region	Variety of dates	Total count (CFU/mg) ± SE		Isolate distribution	
		Fungi	Bacteria	Fungi	Bacteria
Aswan	Aynate	41500^{c}±40.07	75000^{a}±35.36	*A. niger* F4	B1
	Ghazaly	1500^{l}±41.48	12500^{j}±39.13	*A. niger* F4 *A. terreus* F1	B1, B4
	Melkaby	42000^{c}±23.57	30000^{e}±24.98	*A. niger* F4 *Rhizopus* sp. F2 *Penicillium* sp. F3	B1, B2, B3
	Partamoda	26000^{f}±33.00	50000^{c}±33.00	*A. niger* F4	B1, B2, B3, B4
	Shamiya	20000^{k}±21.21	15000^{h}±25.93	*A. niger* F4 *Rhizopus* sp. F2	B1, B4
	Wardy	15000^{i}±37.71	27500^{f}±37.71	*A. niger* F4 *Penicillium* sp. F3	B1, B2, B3
Siwa	Azzawi	22000^{g}±18.86	4500^{k}±20.74	*A. niger* F4 *Rhizopus* sp. F2	B1, B2, B3, B4
	Frehi	40000^{d}±34.41	20000^{g}±34.41	*A. niger* F4 *Rhizopus* sp. F2	B1, B2, B3
	Kaeiby	32000^{e}±31.11	20000^{g}±31.11	*A. niger* F4 *Penicillium* sp. F3	B1, B3, B4
	Shamiya	2500^{h}±42.43	13000^{i}±21.21	*A. niger* F4 *Penicillium* sp. F3 *Rhizopus* sp. F2	B1, B2, B4
El-Wady El-Gadeed	El-Wady I	64000^{b}±28.28	60000^{b}±28.28	*A. niger* F4 *Penicillium* sp. F3 *Circinella* sp. F5	B1, B3
	El-Wady II	80000^{a}±38.18	45000^{d}±37.24	*A. niger* F4	B1, B2

Data were statistically analyzed by one-way analysis of variance (ANOVA). Values are mean of three replicates and given as mean ± standard error (SE). Different letters (a-l) in the same column indicate the range from higher to lower rank as significant differences according to the Student–Newman–Keuls (SNK) test ($P \leq 0.05$).

Microbial pathogens were present in all date fruit samples. These microbes are considered the principal causative agent of the spoilage of date fruits during storage. These findings are compatible with different results for different date varieties grown in other countries [34, 37, 38]. The growth of microorganisms on date fruits is influenced significantly by different factors, including moisture and sugar content [39, 40]. Moisture content is a critical parameter responsible for the microbiological quality of the dates. Shenasi, Aidoo [41] reported for dates from the United Arabe Emirate that microbial counts were high at the first (kimri) and second (rutab) stage of maturation, then decreased significantly at the maturation stage, which had low water activity and high sugar content. Therefore, the microflora in date fruits may depend on the variety and the maturation stage.

The current study indicated that the bacterial counts were higher than the recommended value for food (<10000 CFU/g) [42]. This result means severe bacterial contamination and potential health risks; These results are comparable to previous studies [10, 43]. Similarly, the bacterial load ranged from 4×10^4 to 19×10^4 and from 1×10^6 to 2×10^6 CFU/g, respectively, for hard and soft dates sold in nylon in Nigeria [10]. The authors reported that the bacteria isolated were *Staphylococcus aureus*, *Streptococcus spp.*, *Proteus mirabilis*, *Enterobacter* spp., *Escherichia coli*, and *Salmonella* spp.

Phenotypic characterization of fungal and bacterial isolates

Five morphologically different fungal isolates were isolated from the date varieties (Table 3). Several fungi were identified by examining their characteristics, including colony diameter, color, and colony shape. In addition, microscopic features such as conidial heads, conidiophores, vesicles, and conidia were examined. The morphological features showed variability between the examined fungal isolates (Figure 2A and Table 3). The five fungal isolates examined in this study were found to belong to four different genera *Aspergillus* (*A. niger* F4 and *A. terreus* F1), *Penicillium* sp. (F3), *Rhizopus* sp. (F2) and *Circinella* sp. (F5).

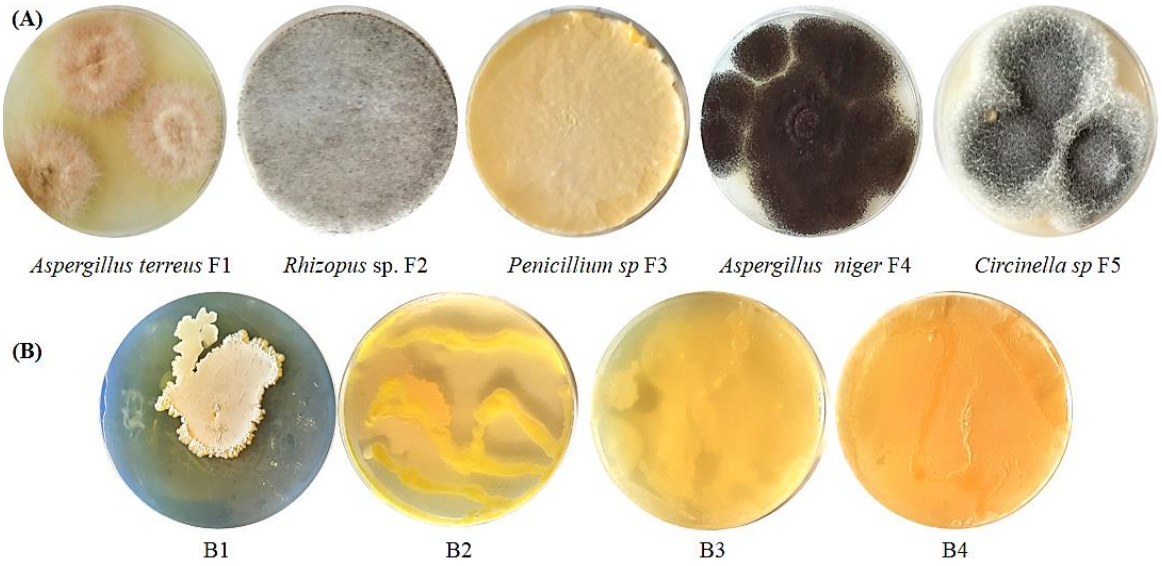

Fig. 2.A: *Colonies morphology of five fungal isolates on the PDA medium after 7 days of incubation at 28 °C.* ***B****: Colonies morphology of four bacterial isolates on NA media after 48 h incubation at 28 °C.*

Table 3. *Morphological characteristics of fungal isolates on the PDA medium at 28°C after 6 days of incubation*

Fungi	Colony diameter (cm)	Colony color	Colony margin	Colony exudates	Colony reverse	Conidial heads	Conidiophores/ Sporangiophores	Vesicles	Conidia
Aspergillus niger F4	5	Black	White to pale yellow	Lacking	Colorless to gray yellow	Large, radiate	Non-septate smooth, hyaline at the base, brownish at the apex	Globose	Brown, rough, globose
Aspergillus terreus F1	4	Gray orange	Thin, broad, whitish	Lacking	Gray orange with yellowish secretion diffusing into medium	Compactly columnar	Non-septate smooth, colorless	Hemispherical,	Globose smooth
Penicillium sp. F3	9	White to cream, yellow	White to cream, yellow	Lacking	White yellow	Mono-verticillate	Septate Smooth colorless	Lacking	Ellipsoid smooth
Rhizopus sp. F2	9	Gray	Gray	Lacking	Gray	Sporangium	Non-septate Sporangiophores rhizoids	Lacking	Brown sporgangiospores
Circinella sp. F5	7	Gray	White to grey	Lacking	Gray	Sporangium	Non-septate Circulate shaped sporangiophores	Lacking	Brown sporgangiospores

Similarly, several fungal isolates were isolated from date palm tissue culture, with *Aspergillus niger*, *Alternaria alternata*, and *Penicillium* spp. as the most prevalent [44]. In addition, out of 360 dried palm fruits collected from Maiduguri city, *Aspergillus niger* recorded the highest incidence [45]. Also, out of twelve date fruit varieties, *Aspergillus niger* was the most predominant fungus [38]. Using 30 samples of Egyptian date fruits incubated on 50% sucrose-Czapek's agar medium at 27°C, Gherbawy collected 29 species and 15 fungal genera [46]. 22.6% of the fungi were found in the genus *Eurotium*, which accounted for 83.3% of all the samples. Compared with some strains, the genus *Eurotium* is represented by 5 species for genotypic analysis. A study by Abu-Zinada and Ali found that different synthetic media significantly impacted the fungal counts per gram of air-dried dates derived from eight local date-palm varieties in Saudi Arabia [47]. Seri and Shakra varieties had the highest fungal counts, whereas Medina had the lowest. *Aspergilus flavus*, *A. niger*, *Penicillium rubrum*, *P. oxalicum*, *Rhizopus stolonifer*, *Stemphylium verruculosum,* and *Fusarium* sp. were generally associated with various date varieties. When the relative humidity increased to 90% at 30 °C and 40 °C, the fungi appeared colonized. In artificial media, glucose concentrations of 60% promoted best growth of the isolated fungi.

A. niger (F4) was the most abundant in all samples, followed by *Rhizopus* sp. (F2), *Penicillium* sp. (F3), *A. terreus* (F1), and *Circinella* sp. (F5), respectively. Generally, *Aspergillus niger* is one of the most common species of the genus *Aspergillus*. Therefore, it is a common food contaminant [48]. In addition, some strains of *A. niger* have been reported to produce potent mycotoxins [49].

Four morphologically different bacterial phenotypes were also isolated and purified on NA plates (Figure 2B). The bacterial isolates were characterized according to their visual and microscopic characteristics (Table 4). One isolate was Gram negative bacilli B1 (monobacilli). The other three isolates were Gram positive, two were found to be bacilli B3 (diplobacilli), and B4 (short rods), and one was found to be staphylococci (B2). Similarly, out of twelve date fruit varieties, *Bacillus* spp., *Staphylococcus aureus,* and *Escherichia coli* were isolated [38].

Table 4. *Morphological characteristics of bacterial isolates on the NA medium at 28 °C after 48 h of incubation*

Bacteria	Culture texture	Culture color	Culture margin	Culture elevation	Culture shape	Cell shape	Gram stain	Spore
B1	Rough	White	Undulate	Flat	Irregular	Monobacilli	-	-
B2	Smooth	Yellow	Irregular	Flat	Circular	Staphylococci	+	-
B3	Smooth	Colorless	Irregular	Flat	Circular	Diplobacilli	+	-
B4	Smooth	Whitish	Entire	Flat	Circular	Short rods	+	-

Genotypic and phylogenetic characterizations of the most abundant fungal and bacterial isolates

In the present investigation, the selected fungal isolate's purified genomic DNA (*A. niger* F4) was prepared and examined by 1% agarose gel electrophoresis. DNA preparations were then used to amplify the ITS regions using the PCR primers ITS1 and ITS4, which resulted in fragments length of 610 bp (Figure 3A). The same primers were then separately used for nucleotide sequencing of the purified DNA fragments. After editing, the obtained sequences were submitted to the GenBank nucleotide database under

the accession number MW811390. The database matching results of the sequences obtained in this study indicated that the examined fungal isolate belongs to *A. niger* F4. Figure 4 shows a neighbor-joining phylogenetic tree constructed based on the alignment of the ITS sequences of *Aspergillus* genotypes using MEGA 6 software. The observed high identity results (99.82%) to database sequences allowed identifying the isolates at the species level.

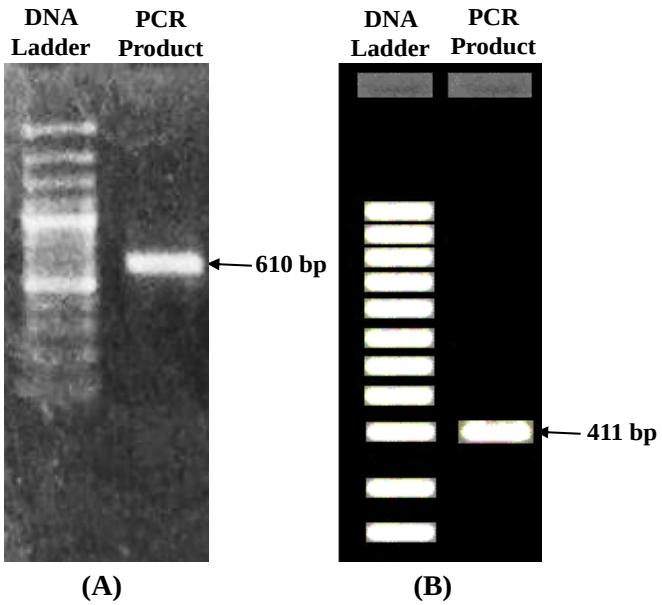

Fig. 3.A: *Agarose gel electrophoresis of the ITS regions amplified using ITS1 and ITS4 PCR primers for the fungal isolate F4. The gel shows (from left to right) GeneRuler 100bp DNA Ladder and the 600 bp fragments of the PCR amplification products.* ***B:*** *Agarose gel electrophoretic profile of the isolate B1 partial 16S rRNA PCR product.*

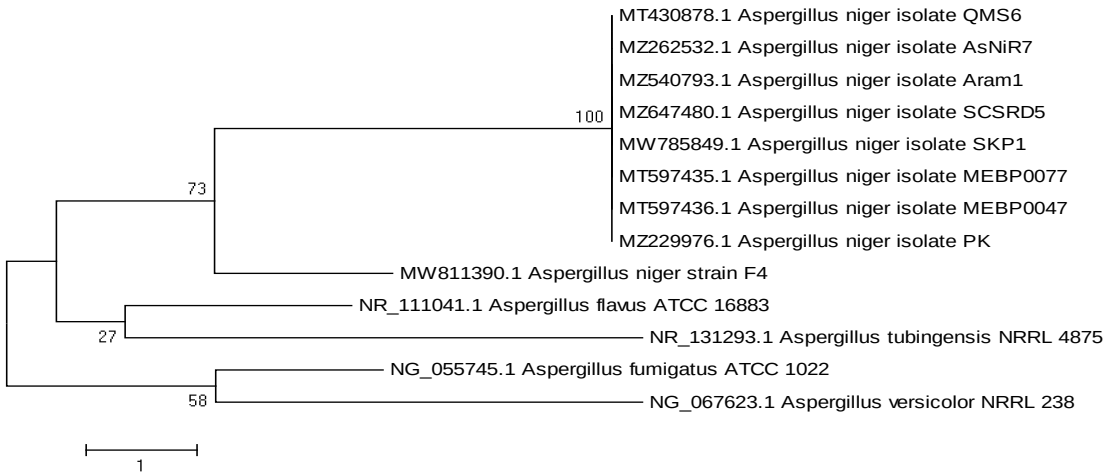

Fig. 4. *A neighbor-joining phylogenetic tree was constructed based on the alignment of the ITS sequences of Aspergillus genotypes using MEGA 6 software. Letters and numbers written before the scientific names are the GenBank accession numbers.*

In addition, the purified genomic DNA of the selected isolate (B1) was prepared and examined by 1% agarose gel electrophoresis. DNA preparation was then used to partially amplify the 16S rDNA regions using the PCR 16S primer pairs, which resulted in fragment length of 411 bp (Figure 3B). The same primers were then separately used for nucleotide sequencing of the purified DNA fragments. After editing, the obtained sequences were submitted to the GenBank nucleotide database under the accession number MW811391. The database matching results of the sequences obtained in this study indicated that the examined bacterial isolate belongs to *Pseudomonas aeruginosa* strain B1. Figure 5 shows the neighbor-joining phylogenetic tree constructed based on the alignment of the 16S rRNA gene sequences of *Pseudomonas* genotypes using MEGA 6 software. The observed high identity results (98.10%) to database sequences allowed identifying the isolates at the species level.

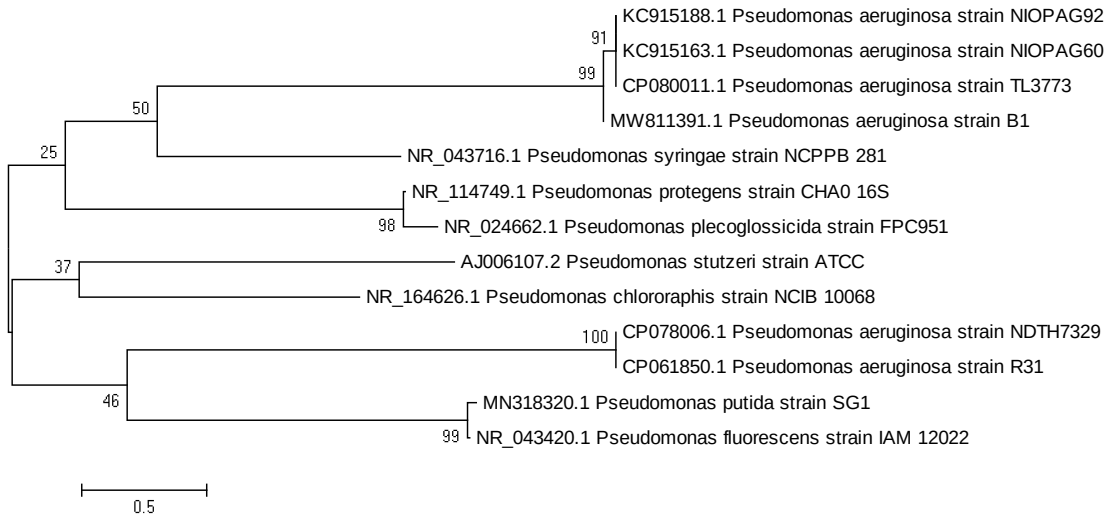

Fig. 5. *A neighbor-joining phylogenetic tree was constructed based on the alignment of the 16S rRNA gene sequences of Pseudomonas genotypes using MEGA 6 software. Letters and numbers written before the scientific names are the GenBank accession numbers.*

Analysis of aflatoxins in dates by LC/MS/MS

The quantitative analysis of the four types of aflatoxins in date samples stored at 25°C and 75% RH for four months was performed by LC/MS/MS. The results shown in Table 5 proved that all types of the tested dates did not contain any detected concentration of the aflatoxins G2, G1, and B2. However, Partamoda and Frehi dates showed low concentrations of the aflatoxin B1 (0.21 and 0.20 μg/kg, respectively). In addition, the date types of Kaeby, El-wady 1and 2 contain a very low concentration of aflatoxin B1<LOQ.

These results agreed with other studies suggesting that most *A. flavus* and *A. niger* isolates could have aflatoxins [50, 51, 52]. In general, the aflatoxins produced by toxigenic strains of *A. flavus*, are amongst the most potent carcinogenic, mutagenic and teratogenic chemicals found in nature [53, 54]. It is known that mycotoxigenic moulds grow and produce four major aflatoxins named B1, B2, G1 and G2. These toxins are often

found in tropical and subtropical climates when suitable environmental conditions are present [55, 56]. Abu-Zinada and Ali [47] found that *A. flavus* was generally associated with some date fruit varieties from Saudi Arabia when the relative humidity (RH) was increased to 90% at 30°C. Grecz et al. also reported that *A. flavus* was one of the natural moulds found on some varieties of Saudi date fruits [57]. It was found that *A. flavus* can be isolated from Egyptian date fruits by treating them with methyl bromide and storing them in polyethylene bags for 8 months at 20-25°C and 60-75% humidity [58]. They found that two isolates produced aflatoxins B_1, G_1 and G_2 in synthetic medium and on dates. Aidoo et al. found *A. flavus* and *A. parasiticus* in some imported date fruits purchased in the UK, but no further investigation of aflatoxin production was carried out [59]. Iqbal, Asi [60] analyzed the aflatoxins in 153 dates and date samples using HPLC equipped with fluorescence detection. The authors found that 38 out of 96 samples (39.6%) of different date varieties and 18 out of 57 (31.6%) samples of date products contained aflatoxins. The total mean level of the aflatoxins ranged between 2.90 to 4.96 μg/kg and 2.76 to 4.80 μg/kg in dates and dates products, respectively. About 16 and 20 samples of dates were found above the acceptable level for aflatoxin B1 and total aflatoxins, respectively (2 μg/kg and 4 μg/kg, respectively).

Table 5. *Aflatoxins detection by LC/MS/MS in different date samples stored at 25 °C and 75% RH for four months*

Date type	Location	Aflatoxins (μg/kg)			
		G2	G1	B2	B1
Aynate	Aswan	ND	ND	ND	ND
Ghazaly	Aswan	ND	ND	ND	ND
Melkaby	Aswan	ND	ND	ND	ND
Partamoda	Aswan	ND	ND	ND	0.21
Shamiya	Aswan	ND	ND	ND	ND
Wardy	Aswan	ND	ND	ND	ND
Azzawi	Siwa	ND	ND	ND	ND
Frehi	Siwa	ND	ND	ND	0.20
Kaeiby	Siwa	ND	ND	ND	<LOQ
Shamiya	Siwa	ND	ND	ND	ND
El-Wady I	El-Wady	ND	ND	ND	<LOQ
El-Wady II	El-Wady	ND	ND	ND	<LOQ

ND: Not detected. Limit of quantification (LOQ) of aflatoxins detected (B1, B2, G1, G2)=0.20 μg/kg.

CONCLUSION

Date fruits are consumed globally in many countries and are mostly loaded with microbial pathogens. Various bacterial and fungal species attack dates cause spoilage during ripening and storage. The present study investigated the current spoilage bacteria and fungi of various postharvest date fruits collected from different Egyptian geographical regions and shared in establishing a bacterial and fungal profile of fruits. In addition, the isolation and identification of dates-borne microbes based on the morphological identification and molecular identification of the most common microbes were studied. The microscopic examination of the infected date samples showed various fungal structures (hyphae, spores, conidial head, etc.). In addition, all fungal structures observed in the infected specimens belong to the fungus of *A. niger*. The total viable fungi counts in twelve date variety samples ranged from 1500 to 80000 CFU/g after 6 days of incubation on the PDA medium. Five morphologically different fungal isolates (*A. niger*

F4, *A. terreus* F1, *Penicillium* sp. F3, *Rhizopus* sp. F2, and *Circinella* sp. F5) were isolated from the date samples. *A. niger* F4 was the most abundant in all samples. Similarly, the total viable counts of bacteria in twelve date varieties samples ranged from 4500 to 75000 CFU/g after 48 h incubation on the NA medium. Four morphologically different bacterial phenotypes (B1, B2, B3, and B4) were isolated and purified on the NA plates. *Bacillus sp.* B1 was the most abundant bacterial isolate in all samples. Aflatoxins G2, G1, and B2 were not detected in all date varieties; however, Aflatoxin B1 was detected in two varieties, Partamoda and Frehi.

Abbreviations. ***A. niger***: *Aspergillus niger*; **ANOVA**: Analysis of variance; **BLASTn**: nucleotide basic local alignment search tool; **HPLC/MS**: High pressure liquid chromatography/mass spectrometer; **NA**: nutrient agar; **NB**: nutrient broth; **NCBI**: National Centre for Biotechnology and Information; ***P. aeruginosa***: *Pseudomonas*; *aeruginosa*; **PDA**: potato dextrose agar; **PDB**: potato dextrose broth; **SNK**: Student-Newman-Keuls.

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